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Identification of a novel locus on chromosome 2q13 which predisposes to clinical vertebral fractures independently of bone density

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ABSTRACT

Objectives: To identify genetic determinants of susceptibility to clinical vertebral fractures, the most common complication of osteoporosis. **Methods:** Genome-wide association was carried out in 1,553 postmenopausal women with clinical vertebral fractures and 4,340 controls, with replication in 667 cases and 2,105 controls. Potentially causal variants were identified using eQTL data from transiliac bone biopsies and bioinformatic studies. **Results:** A locus tagged by rs10190845 was identified on chromosome 2q13 which was significantly associated with clinical vertebral fractures ($p=1.27x10^{-8}$) with a large effect size (odds ratio 1.75, 95% CI 1.4-2.1). Three other suggestive loci were identified on chromosomes 1p31, 11q12 and 15q11. All were novel and had not previously been associated with BMD or clinical fractures. Analysis of variants that have been associated with spine BMD or fractures in previous studies identified eight that were significantly associated with clinical vertebral fractures in this study. Bioinformatic analysis identified several potentially functional SNPs which were associated with expression of the positional candidate genes *TTL* and *SLC20A1*. **Conclusion:** We have identified a novel genetic variant with one of the largest effect sizes so far reported in the field of osteoporosis genetics that is associated with clinical vertebral fractures by mechanisms that are independent of BMD. Further studies are in now progress to evaluate the mechanism that underlies this association.

INTRODUCTION

Osteoporosis is a common disease with a strong genetic component. It is characterised by low bone mineral density (BMD), deterioration in the microstructural architecture of bone and an increased risk of fragility fractures. Vertebral fractures are the most common complication of osteoporosis [1]. They are characterised by loss of height and deformity of the affected vertebrae and are associated with an increased risk of other fractures [2]. Vertebral fractures are commonly overlooked since only 8-30% of patients with radiological evidence of vertebral fractures come to medical attention [3,4]. About one-third of patients with radiographic evidence of vertebral fractures also present clinically [5,6] with symptoms such as back pain, kyphosis and height loss [7]. These patients are said to have clinical vertebral fractures. Clinical vertebral fractures are associated with a markedly increase risk of future fractures and increased mortality, indicating their importance as a marker of poor clinical outcome in osteoporosis [8].

Over recent years, major advances have been made in understanding the genetic susceptibility to osteoporosis through genome-wide association studies (GWAS) [9-18] and genome sequencing [19,20]. To date, these studies have identified 62 loci that are associated with BMD at a genome-wide significant level and 14 loci that are associated with clinical fractures. In contrast, much less is known about genetic determinants of vertebral fracture. A previous genome-wide association study published by Oei and colleagues involving a discovery cohort of 8,717 subjects with morphometric vertebral deformities and 21,793 controls failed to identify any significant genetic predictors of vertebral fracture at a genome-wide significant level [21]. A limitation of this study was the diversity of methods and criteria used to define the presence of vertebral deformities in different cohorts [21] and the lack of a consensus on what constitutes a true vertebral fracture on imaging [22]. In view of these issues, the aim of this study was to perform a genome-wide association study to identify genetic variants that predisposed to clinical vertebral fractures.

PATIENTS AND METHODS

The study involved a discovery phase involving 1,553 clinical vertebral fracture cases and 4,340 controls and a replication sample of 667 cases and 2,105 controls as summarise in supplementary Table S1. The genome wide association study was performed using standard methodology as detailed in the supplementary Text S1.

RESULTS

Characteristics of the study populations

The mean (± standard deviation) age of the patients with clinical vertebral fractures was 71.3 ± 9.3 years with a bone mineral density T-score at the lumbar spine of -2.72 ± 1.4; and at the femoral neck of -2.57 ± 1.1. The controls were not matched with the cases by age and did not undergo phenotyping for vertebral fracture on the basis that clinical vertebral fractures are uncommon in the general population (estimated incidence of 9.8/1000 person-years in 75-84 year olds) [23]. While it is possible that clinical vertebral fractures may have occurred in some controls in later life this is unlikely to have substantially affected the results of the analysis [24]. This approach has been used previously for genome-wide studies in various common diseases including diabetes, Paget's disease, and rheumatoid arthritis [25,26].

Genome-wide association analysis

Since different genotyping platforms were used in the different cohorts (see supplementary information page 4 for further details), association analysis was conducted following imputation of all genotypes into the CEU panel of HapMap II reference (see Patients and Methods section). Following imputation, we analysed 2,366,456 SNPs and identified 31 with suggestive evidence of association with vertebral fracture ($p \leq 10^{-4}$). Details are summarised in supplementary Table 2, and the Manhattan and quantile-quantile plots from the discovery sample are shown in supplementary Figures 1 and 2. Each study was corrected by genomic control, and genomic inflation factors ranged between $\lambda = 1.001$ to $\lambda = 1.046$ for genotyped SNPs and $\lambda = 1.006$ to $\lambda = 1.036$ after imputation.

Replication analysis

We analysed the 31 suggestively associated SNPs identified in the discovery cohort (supplementary Table 2) and seven additional SNPs that had been significantly associated with clinical fractures in a previous GWAS (supplementary Table 3) [10]. The combined discovery and replication analysis corrected for age identified one SNP (rs10190845) on chromosome 2q13 with genome-wide significant evidence of association with clinical vertebral fractures ($p = 1.27 \times 10^{-8}$). The predisposing allele had a frequency of 0.034 in cases compared with 0.022 in controls and the odds ratio for susceptibility to fracture was 1.75 [95% CI: 1.44-2.12] (Figure 1). The results were similar without age correction ($p=4.9 \times 10^{-8}$; odds ratio 1.66 [95% CI: 1.38 – 1.99]). Three other SNPs on chromosomes 1p31, 11q12 and 15q11 were suggestively associated with vertebral fracture (Table 1 and supplementary Figure 3). None of these regions have previously been found to be associated with BMD or fracture in other GWAS [10,13] .

The top hit maps to a region which contains eleven potential candidate genes (Figure 2). This region has previously been implicated as a genetic regulator of bone density by Estrada and

colleagues [10] who reported that rs17040773 within *ANAPC1* was associated with femoral neck BMD ($p = 1.5 \times 10^{-9}$), but not with clinical fractures ($p = 0.79$). The rs17040773 was not in linkage disequilibrium with rs10190845 ($r^2=0.006$), and, when we performed conditional analysis on rs17040773, we found that rs10190845 remained significantly associated with clinical vertebral fractures ($p= 2.09 \times 10^{-8}$; odds ratio 1.73 [95% CI: 1.43-2.09]). This suggests that rs10190845 constitutes an independent signal which predisposes to vertebral fracture by mechanisms that are independent of an effect on BMD.

Table 1. Variants showing suggestive or significant association with vertebral fracture

				Discovery (n = 5,893)			Replication (n= 2,772)			Combined (n= 8,665)			
Chr	**SNP**	**Position**	**A**	**AF**	**p**	**OR (95% CI)**	**AF**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**I^2**	**I^2 p**
2	rs10190845	112666992	A	0.03	$2.4x10^{-5}$	1.70 (1.33-2.17)	0.05	$1.60x10^{-4}$	1.84 (1.34-2.53)	$1.27x10^{-8}$	1.75 (1.45-2.12)	5.9	0.39
11	rs7121756	57504473	A	0.29	$5.2x10^{-5}$	1.22 (1.11-1.35)	0.28	0.01102	1.23 (1.05-1.45)	$1.27x10^{-6}$	1.23 (1.13-1.33)	0.0	0.67
15	rs2290492	90808978	A	0.23	$3.4x10^{-5}$	1.24 (1.12-1.37)	0.21	0.0214	1.23 (1.03-1.46)	$1.61x10^{-6}$	1.24 (1.13-1.35)	53.7	0.02
1	rs1360181	68486723	T	0.84	$8.4x10^{-5}$	0.80 (0.71-0.89)	0.83	0.00831	0.77 (0.64-0.93)	$1.87x10^{-6}$	0.79 (0.71-0.87)	7.7	0.57

The allele (A) and allele frequency (AF) for each of the variants is shown along with the p value for association, odds ratio (OR) and 95% confidence interval (95% CI). The values shown are adjusted for age but similar results were obtained for unadjusted association tests.

Functional evaluation of chromosome 2q13 locus

In order to gain insight into the functional basis of the association at 2q13 we used SuRFR [27] which integrates functional annotation and prior biological knowledge to identify potentially causal genetic variants. This analysis focused on a linkage disequilibrium block of approximately 700kb surrounding the top hit rs10190845. We identified a total of 936 SNPs within the region which were analysed in the GWAS (n=376) or which were in linkage disequilibrium (r^2 value of > 0.7) with rs10190845, or which showed suggestive association to vertebral fractures ($p < 5 \times 10^{-3}$). We imputed the genotypes for the SNPs within the region of interest using the 1000 Genomes phase 3 panel as reference and tested the SNPs for association with clinical vertebral fractures. We removed 878 of the SNPs since they showed no association with clinical vertebral fractures in our dataset. The remaining 58 candidate SNPs were tested for association with the level of expression of genes within the candidate locus using a bone-derived gene expression dataset (eQTLs) [28] (Tables 2 and 3 and supplementary Figure 4). This resulted in the identification of nine SNPs which were eQTLs for genes within the region. These SNPs were analysed by SuRFR along with the top hit rs10190845 (Table 2 and supplementary Figure 4).

The top ranking variant identified by SuRFR, rs35586251, located within exon 3 of *FBLN7* is a non-synonymous substitution (p.Val119Met). However, analysis using various *in silico* software tools yielded inconsistent results with regard to functionality of this SNP at the protein level (supplementary Table 4). The other SNPs ranked as potentially functional by SuRFR were associated with expression of *TTL*, *SCL20A* or both genes. The top ranking functional variant rs35586251 was associated with increased expression of *TTL* ($p=6.6 \times 10^{-6}$). Four other variants were also associated with increased expression of *TTL* and reduced expression of *SLC20A1* (p-values ranging from 2.1×10^{-6} to 10^{-5}). The second ranking variant, rs77172864, in strong LD with the GWAS top hit ($r^2=0.79$), was associated with reduced expression of *SLC20A1* ($p = 10^{-4}$) (Tables 2 and 3).

Table 2. Functionality of SNPs in 2q13 region, predicted by SuRFR

Rank	SNP ID	R^2 with rs10190845	A (AF)	GWAS p-value	OR (95%CI)	Location	GERP Value	DNase HS sit	DNase Foot	Ernst Score	Position Score	MAF Score	Enhancer score	TFBS score	Total score	eQTL	eQTL gene(s)	eQTL p
1	rs35586251	0.17	A (0.02)	$2.09x10^{-4}$	1.69 (1.28-2.24)	*Exon FBLN7*	4.47	0	0	7	5	0.02	0	0	9.89	Yes	*TTL*	6.6×10^{-6}
2	rs77172864	0.79	G (0.03)	$4.96x10^{-5}$	1.68 (1.31-2.17)	Intergenic	0.18	0	0	1	3	0.02	0	0	8.56	Yes	*SCL20A1*	0.0001
3	rs10190845	1	A (0.03)	$2.4x10^{-5}$	1.70 (1.33-2.17)	Intergenic	0	0	0	2	3	0.96	0	0	8.06	No	-	-
4	rs77996972	0.22	T (0.02)	$2.11x10^{-4}$	1.69 (1.28-2.23)	Intron *FBLN7*	1.77	313	0	7	1	0.02	0	0	7.61	Yes	*TTL* *SLC20A1*	3.8×10^{-6} 5.5×10^{-5}
5	rs75814334	0.22	T (0.02)	$2.11x10^{-4}$	1.69 (1.28-2.23)	Intron *FBLN7*	0.43	239	0	8	1	0.02	0	0	7.56	Yes	*TTL* *SLC20A1*	2.1×10^{-6} 6.6×10^{-5}
6	rs74792868	0.22	A (0.02)	$2.1x10^{-4}$	1.69 (1.28-2.24)	Intron *FBLN7*	0	0	0	9	1	0.02	0	0	7.5	Yes	*TTL* *SLC20A1*	2.0×10^{-5} 2.8×10^{-5}
6	rs72943913	0.29	G (0.03)	$5.48x10^{-5}$	1.67 (1.30-2.14)	Intron *ZC3H8*	0.15	0	0	3	1	0.02	0	0	6.46	Yes	*SLC20A1*	0.0001
7	rs112275607	0.22	A (0.02)	$2.13x10^{-4}$	1.69 (1.28-2.24)	Intron *FBLN7*	0	0	0	8	1	0.02	0	0	6.83	Yes	*TTL* *SLC20A1*	2.8×10^{-6} 6.2×10^{-5}
8	rs113085288	0.06	T (0.02)	$1.79x10^{-4}$	1.70 (1.29-2.24)	Intron *FBLN7*	0	0	0	7	1	0.02	0	0	6.08	Yes	*SLC20A1*	4.1×10^{-6}
9	rs113428223	0.29	T (0.03)	$4.55x10^{-5}$	1.70 (1.31-2.20)	Intron *ZC3H6*	0	0	0	2	1	0.02	0	0	5.61	Yes	*SCL20A1*	0.0001

A (AF): allele (allele frequency); GERP: Genomic evolutionary rate profiling; DNAase HS: DNase hypersensitivity; DNase foot: DNase footprint; Ernst score: classes of chromatin states (recurrent combinations of chromatin marks); MAF: minor allele frequency; TFBS: transcription factor binding site.

Table 3. Correlation between genotypes for potentially functional SNP and bone-specific expression of genes in the candidate region

RANK	SNP	GENE	PROBE	A1	A2	FRQ	BETA	SE	P
1	rs35586251	*TTL*	224896_s_at	A	G	0.017	0.65	0.13	$6.62x10^{-6}$
2	rs77172864	*SLC20A1*	230494_at	G	A	0.013	-0.46	0.11	0.00011
4	rs77996972	*TTL*	224896_s_at	T	C	0.012	0.67	0.13	$3.80x10^{-6}$
		SLC20A1	230494_at	T	C	0.012	-0.49	0.11	$5.50x10^{-5}$
5	rs75814334	*TTL*	224896_s_at	T	C	0.013	0.67	0.13	$2.10x10^{-6}$
		SLC20A1	230494_at	T	C	0.013	-0.48	0.11	$6.60x10^{-5}$
6	rs74792868	*TTL*	224896_s_at	A	G	0.012	0.66	0.14	$2.00x10^{-5}$
		SLC20A1	230494_at	A	G	0.012	-0.53	0.12	$2.80x10^{-5}$
6	rs72943913	*SLC20A1*	230494_at	G	A	0.013	-0.46	0.11	0.00011
7	rs112275607	*TTL*	224896_s_at	A	G	0.013	0.67	0.13	$2.80x10^{-6}$
		SLC20A1	230494_at	A	G	0.013	-0.48	0.11	$6.02x10^{-5}$
8	rs113085288	*SLC20A1*	230494_at	T	A	0.008	-0.72	0.14	$4.06x10^{-6}$
9	rs113428223	*SLC20A1*	230494_at	T	C	0.013	-0.46	0.11	0.0001

The data shown are only for the associations which were significant after Bonferroni correction (p value for significance $\leq$0.0002). A1: allele 1, A2: Allele 2, FRQ: frequency of allele 1, BETA: effect size on regression analysis referred to A1 allele, SE: standard error of beta estimate, probe IDs obtained from the Affymetrix HG U133 2.0 plus array.

Association between clinical vertebral fractures and other osteoporosis related phenotypes

In order to determine if there was overlap between the SNPs identified as associated with lumbar spine BMD in previous GWAS with those associated with clinical vertebral fracture in this study, we evaluated 50 SNPs that have been associated with lumbar spine BMD at a genome-wide significant level in previous studies in our dataset [10,11,13,19,20]. This resulted in the identification of four variants that were nominally associated with clinical vertebral fracture after Bonferroni correction (Table 4). Of the 15 variants previously associated with clinical fracture [10,13], three were associated with clinical vertebral fractures in this study. We also analysed the SNPs identified by Nielson and colleagues (Nielson et al., 2016, unpublished) as genome-wide significant predictors of volumetric vertebral bone mineral density for association with clinical vertebral fractures in our dataset. Of the six genome-wide significant SNPs identified in Nielson et al, we found that one was significantly associated with clinical vertebral fractures after Bonferroni correction (rs12742784, $p=6.24 \times 10^{-5}$).

Table 4. Association between known genetic determinants of spine BMD and clinical vertebral fractures in the combined GWAS dataset.

Previous studies								Present study	
Study	SNP	locus	Candidate gene	Phenotype	Allele	beta	p	beta	p
Estrada	rs1346004	2q24.3	GALANT3	LS-BMD	A	-0.06	$3.87x10^{-30}$	+0.16	0.0002
Estrada	rs4727338	7q21.3	SLC25A13	LS-BMD	C	+0.07	$2.13x10^{-35}$	-0.15	0.0004
Estrada	rs6426749	1p36.12	ZBTB40	LS-BMD	C	+0.1	$1.86x10^{-44}$	-0.22	0.0003
Styrkarsdottir	rs7524102	1p36	WNT4	LS-BMD	A	-0.11	$9.2x10^{-9}$	+0.23	0.0002
Estrada	rs4727338	7q21.3	SLC25A13	Clinical fracture	G	+0.08	$5.9x10^{-11}$	+0.14	0.0004
Estrada	rs6426749	1p36.12	ZBTB40	Clinical fracture	G	+0.07	$3.6x10^{-6}$*	+0.22	0.0003
Estrada	rs6959212	7p14.1	STARD3NL	Clinical fracture	T	+0.05	$7.2x10^{-5}$*	+0.15	0.001
Nielson	rs12742784	1p36.12	ZBTB40	Vertebral BMD	T	+0.09	$1.05x10^{-10}$	-0.20	$6.24x10^{-5}$

The variants shown are those that were significant after Bonferroni correction for testing 56 BMD variants (p threshold for association 0.0009) and 16 fracture variants (p threshold for association 0.003). *SNP significantly associated with clinical fracture after Bonferroni correction (p threshold at Estrada et al $5x10^{-4}$).

DISCUSSION

Many advances have been made in defining the genetic determinants of bone mineral density and fractures through large scale genome-wide association studies, genome sequencing studies and linkage studies in rare bone diseases [29]. For example, linkage studies have shown that loss-of-function and gain-of-function variants in *LRP5* cause early onset osteoporosis [30] and high bone mass [31] respectively, whereas loss of function mutations affecting *SOST* and *LRP4* have been identified as causes of high bone mass and osteosclerosis [32,33]. Genome-wide association studies and genome sequencing studies have also been successful in identifying multiple loci that regulate bone mineral density [9-11,19] and a smaller number that predispose to clinical fractures [10,19].

Although vertebral fractures are one of the most common and important complications of osteoporosis, relatively little is known about their genetic basis [34]. In a previous study of 8,717 cases and 21,793 controls, Oei and colleagues failed to identify any locus with significant evidence of association with morphometric vertebral deformities [21]. In the present study however, we identified one significant and several suggestive loci that predisposed to clinical vertebral fractures. Most likely the differences between these studies are due to the fact that clinical vertebral fractures are a more clearly defined phenotype than morphometric vertebral deformities [22]. The genome-wide significant SNP identified in the present study, rs10190845, maps to chromosome 2q13, a region previously associated with low femoral neck bone density [10]. However, when conditioning on rs17040773, the previously reported top SNP at the locus [10], the association with rs10190845 remained significant, indicating that it is a novel signal.

In order to determine if there was an overlap between the results of this study and those previously reported, we analysed 51 SNPs that have previously been associated with either spine BMD or clinical fractures and identified seven variants that were significantly associated with clinical vertebral fracture in this study. The SNPs that were associated with low BMD in previous studies were associated with an increased risk of clinical vertebral fractures in this study and those associated with an increased risk of clinical fractures in previous studies were associated with an increased risk of clinical vertebral fractures in this study.

Furthermore, when we analysed six SNPs that were significantly associated with vertebral bone mineral density on quantitative CT analysis (Nielson et al., 2016, unpublished) we identified one locus on chromosome 1p36, close to *ZBTB40*, that was significantly associated with clinical vertebral fracture in this study. These results support the importance of *ZBTB40*

as a predictor of clinical fractures and suggest that the mechanism of association is most probably mediated by changes in BMD. The observations in this study, when taken together with the findings of Nielson and Estrada [10] indicate that there is a partial overlap between loci that regulate lumbar spine BMD, and clinical vertebral fractures, but that there are some genetic determinants of clinical vertebral fracture which are unique and which operate independently of BMD.

In order to identify the mechanisms by which 2q13 predisposes to vertebral fracture we conducted functional and bio-informatic studies to determine if rs10190845 or other SNPs nearby were likely to be functional variants. These studies identified several potentially functional SNPs in the same LD block as rs10190845, which might account for the association we observed. The top ranking SNP from SuRFR analysis was rs35586251, which was strongly associated with expression of the *TTL* gene within the candidate locus (supplementary Figure 5). However, the second ranking SNP, rs77172864, was significantly associated with the expression of *SLC20A1*. Several other SNPs were also significantly associated with expression of *TTL* and/or *SLC20A1,* raising the possibility that alterations in expression of one or both genes might account for the predisposition to vertebral fractures.

The *TTL* gene encodes a tubulin tyrosine ligase involved in regulation of the cytoskeleton. Previous studies have shown that *TTL* is involved in neuronal development [35] and injury signalling [36], raising the possibility that variants that regulate *TTL* might be involved in regulating pain perception, which could account for the fact that predisposing variants have not previously been associated with BMD. Other mechanisms might also be possible and further studies need to be performed in order to address the role of *TTL* in vertebral fracture.

The other main candidate gene, *SLC20A1*, encodes Pit1, which facilitates the entry of inorganic phosphate into the cytoplasm [37]. Previous studies have shown that *SLC20A1* is involved in mineralisation [38-41], which raises the possibility that differences in expression of this gene could be involved in regulating susceptibility for vertebral fractures by an effect on bone mineralisation. However, *SLC20A1* has not been identified previously as a predictor of BMD or fractures suggesting that an alternative mechanism may be operative, or that *TTL* rather than *SLC20A1* is the candidate gene within the 2q13 locus.

Limitations of the study include the fact that the total sample size was relatively small and the power to detect alleles of modest effect size was limited. It's possible that we may have missed associations between rare variants and clinical vertebral fractures since the imputation we performed was against HapMap reference panel. Although case definition was clinically based there was no significant heterogeneity in the associations we observed across centres.

A strength of the present study is that it has provided important new information on the genetic determinants of clinical vertebral fracture. Although clinical vertebral fractures are a hallmark of osteoporosis, it is of great interest that the top hit and other suggestive hits acted independently of BMD. This suggests that the variants identified might be acting as markers for perception of pain or other factors that are associated with the clinical presentation of vertebral fractures. We also found that some of the variants previously identified as regulators of spine BMD were associated with clinical vertebral fractures in this study but with effects that were weaker than the top hit and other suggestive hits. Taken together, the data suggest that the genetic basis of clinical vertebral fracture is complex involving variants that act independently of BMD as well as those that are associated with spine BMD. Further research is now warranted to fully investigate the mechanisms involved.

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AUTHOR CONTRIBUTION

Study conception: SHR, NA, AGU, FR; data collection: NA, OMEA, LM, MLB, PR, AD, JMO, CV, JC, JAR, LBH, BLL, MAB, ELD, SK, K-TK, RU-M, JdP, RG-S, JRL, RLP, PD'A, NG-G, XN, SM-B, JM, OW, JE, BF, MM, KES, PN, JFW, GD, JS, ID, TT, LF, FG, LG, GL, RE; Genotyping: AGU, FR; data analysis: NA, OMEA, SR, OKO, KMG, NMR, KLE, CMN, H-YH, DK, XL, BF, MM, KE, LH, LO, CM-G; drafting of the manuscript: NA and SHR; all authors contributed to critically review the article and approved the final manuscript. NA, SR and NMR takes responsibility for the data analysis.

COMPETING INTERESTS

None declared

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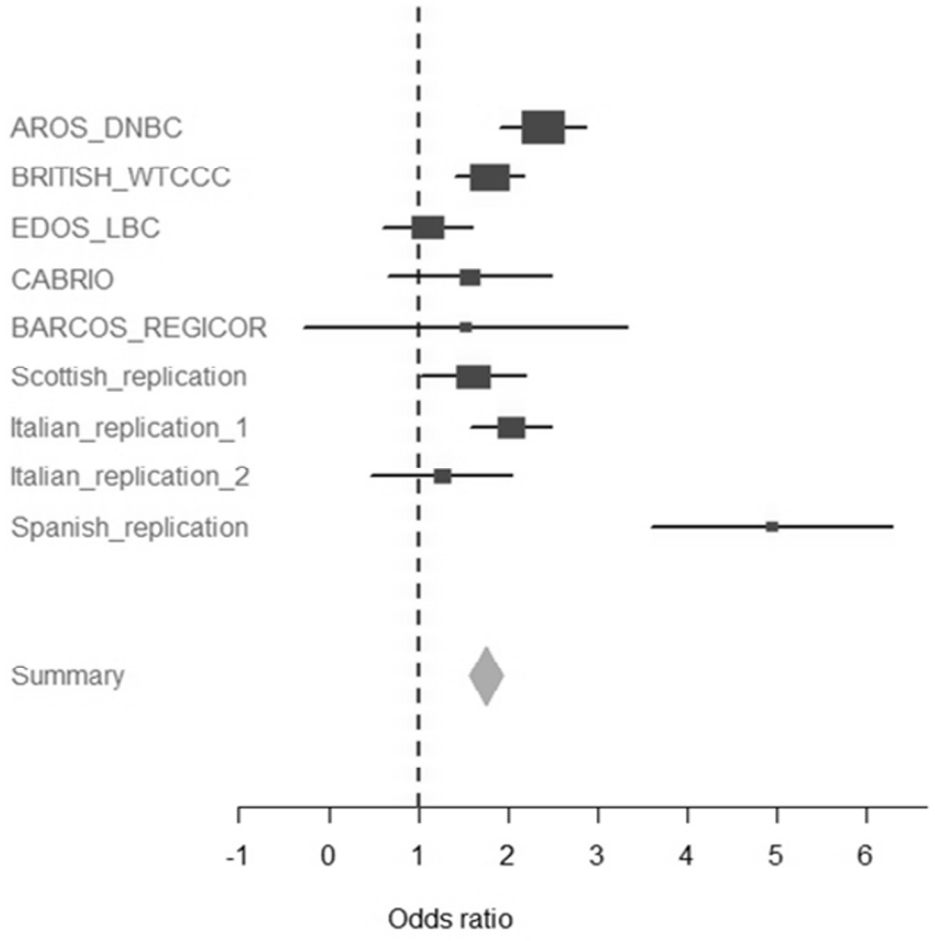
Fig 1. Cohort specific association between rs10190845 and clinical vertebral fracture

The point estimates (squares) and 95% confidence intervals (horizontal lines) for individual studies are shown with the summary indicated by the diamond using a fixed effect model. "Italian_replication_1" study corresponds to Florence-InCHIANTI cohorts and "Italian_replication_2" study comprises the Turin and Siena cohorts. Cohort sizes are reflected by square dimensions.

Fig 2. Regional association plot of 2q11 susceptibility locus for clinical vertebral fracture

The SNPs are colour coded according to the extent of LD with the SNP showing the highest association signal from the combined analysis (represented as a purple diamond). The estimated recombination rates (cM/Mb) from HapMap CEU release 22 are shown as light blue lines, and the blue arrows represent known genes in the region. The red line shows the threshold for genome-wide significance ($p = 5 \times 10^{-8}$).



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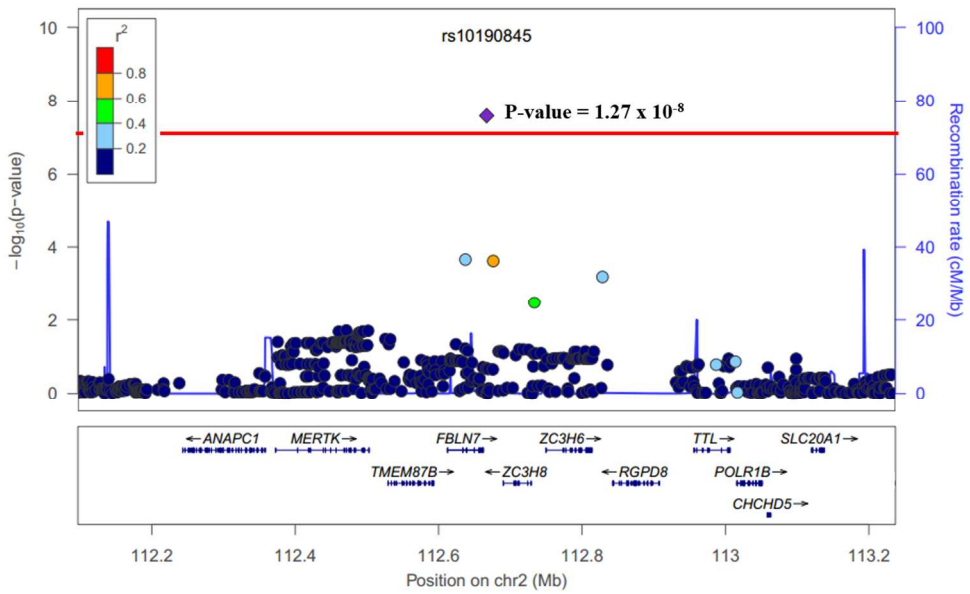
154x161mm (111 x 106 DPI)



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236x140mm (150 x 150 DPI)

Supplemental data

Identification of a novel locus on chromosome 2q13 which predisposes to clinical vertebral fractures independently of bone density

Nerea Alonso, Karol Estrada, Omar ME Albagha, Lizbeth Herrera, Sjur Reppe, Olof K Olstad, Kaare M Gautvik, Niamh M Ryan, Katheryn L Evans, Carrie M Nielson, Yi-Hsiang Hsu, Douglas P Kiel, Bjarke Feenstra, Xueping Liu, Mads Melbye, Laura Masi, Maria Luisa Brandi, Philip Riches, Anna Daroszewska, Jose M Olmos, Carmen Valero, Jesus Castillo, Jose Antonio Riancho, Lise B Husted, Bente L Langdahl, Matthew A Brown, Emma L Duncan, Stephen Kaptoge, Kay-Tee Khaw, Ricardo Usategui-Martin, Javier del Pino, Rogelio Gonzalez-Sarmiento, Joshua R Lewis, Richard L Prince, Patrizia D'Amelio, Natalia Garcia-Giralt, Xavier Nogues, Simona Mencej-Bedrac, Janja Marc, Orit Wolstein, John Eisman, Ling Oei, Carolina Medina-Gomez, Katharina E Schraut, Pau Navarro, James F Wilson, Gail Davies, John Starr, Ian Deary, Toshiko Tanaka, Luigi Ferrucci, Fernando Gianfrancesco, Luigi Gennari, Gavin Lucas, Roberto Elosua, Andre G Uitterlinden, Fernando Rivadeneira, Stuart H Ralston.

INDEX

PATIENTS AND METHODS

Phenotype definition

The case definition of clinical vertebral fractures was that the patient had presented to primary or secondary care with symptoms of acute or chronic back pain, height loss, and kyphosis, with evidence of vertebral fractures on imaging that were considered to account for the symptoms. Patients with secondary causes of osteoporosis such as premature menopause (less than 45-years old), osteogenesis imperfecta, rheumatoid arthritis, inflammatory bowel disease, malabsorption, myeloma, thyrotoxicosis, and primary hyperparathyroidism were excluded as well as patients who had previously been or were currently being treated with corticosteroid therapy (prednisolone > 7.5 mg/day or equivalent for more than 3 months). The controls were selected on the basis that they came from the same geographical region as the vertebral fracture cases and that DNA samples and/or genome wide association data were available. Controls did not undergo phenotyping for the presence of vertebral fractures or vertebral deformities under the rationale that clinical vertebral fractures are relatively uncommon in the general population and that misclassification of the control group with undiagnosed cases is unlikely to influence the results of the analysis substantially, other than to have a minor effect on study power.

Discovery Cohort

Patients with clinical vertebral fractures included in the discovery cohort (n=1,553) were derived from nine centres. Five centres recruited patients from outpatient clinics for the treatment of osteoporosis. These were based in Edinburgh, United Kingdom (371); Aarhus, Denmark (n=299); Barcelona (n=72) and Santander (n=256), Spain; and Ljubljana, Slovenia (n=40). Additional patients with clinical vertebral fractures in the discovery cohort were derived from the CAIFOS study (n=98), a randomised trial of calcium supplementation in ambulatory women older than 70 years [1]; the Anglo-Australasian Osteoporosis Genetics Consortium Australia (AOGC), a population-based, clinic/hospital-based cohort, including 2073 women from Australia and the United Kingdom (n=234) [2]; the Dubbo Osteoporosis Epidemiology Study (DOES), a population-based study including women aged 60 years or older, from Dubbo, New South Wales, Australia (n=33) [3]; and the European Prospective Investigation of Cancer, Norfolk study (EPIC-Norfolk), a population-based study involving over 30,000 people living in Norfolk, UK, aged between 40-79 (n=150) [4].

Cases and controls were sampled from the same geographical region and same ethnic background. Santander and Ljubljana recruited controls locally. The controls for Aarhus cases were subjects from Danish National Birth Cohort (DNBC) a population based sample

of 100,000 pregnant women from all regions in Denmark [5] . The controls for Spanish cases from Barcelona were subjects from Registre Gironi del Cor (REGICOR), a population based study of cardiovascular disease in Spain [6]. The controls for cases of British descent from Edinburgh, EPIC, AOGC, CAIFOS and DOES were derived from the Wellcome Trust Case Control Consortium (WTCCC2) [7] or the 1921 and 1936 Lothian Birth Cohorts [8].

Replication Cohort

Clinical vertebral fracture cases for the replication cohort were derived from clinic referrals to Edinburgh, United Kingdom (n=236); Florence (n=282), Turin (n=86), Italy; and Salamanca, Spain (n=63). Relevant clinical details of the patients from each centre are shown in supplementary Table S1. The controls for the Edinburgh cases were derived from the Orkney Complex Disease Study (ORCADES), a cohort study from the Orkney Islands, an isolated population in Scotland, UK. The controls for the Italian cases were from the Invecchiare nel Chanti (InCHIANTI) cohort, a population-based study of older people from the Chianti area in Tuscany, Italy [9]; and the SIENA cohort, a study of postmenopausal women recruited from primary care registers of Siena residents and participating in an epidemiological survey on risk factors for osteoporosis. Controls were locally recruited for the Salamanca samples.

Ethical Approval

Written informed consent was obtained from all individuals participating in the studies in the various centres and each study received local ethical approval by the relevant ethics committee.

Genotyping and quality control

A variety of genotyping platforms were used. For the discovery cohorts, the AOGC cases were genotyped with a variety of Illumina arrays (arrays including the Illumina Infinium II, the Illumina HumHap300; and the 370 CNVDuo and Quad arrays) at the University of Queensland Diamantina Institute as previously described [2]. The remainder of the discovery cases were genotyped using the Illumina OmniExpress array at the Genetic Laboratory of the Erasmus Medical Centre in Rotterdam. For the control cohorts, the DNBC samples were genotyped using the Illumina 660w Quad v1.4 array; the REGICOR with an Affymetrix 6.0 array; the WTCCC2 using an Illumina 1.2M custom array; the Lothian Birth Cohort using the Illumina 610 Quad array; the Cantabria, and Slovenia controls using the Illumina OmniExpress array.

Standard quality control measures were performed using PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/) [10]; SNPs with low call rate (<95%), those that

deviated from Hardy-Weinberg equilibrium ($p < 10^{-7}$), those with minor allele frequency < 0.01 and missingness χ^2 $p < 10^{-7}$ were excluded. Samples with low quality (call rate < 97.5%), excess heterozygosity, gender mismatch, non-European ancestry and cryptic family relatedness were also removed from the final analysis (values deviated more than 4 times the standard deviation from the mean). The number of cases and controls after quality control are listed in supplementary Table 1. Genotyping cluster plots for significant and suggestive SNPs ($p < 5 \times 10^{-5}$) were visually inspected and only the SNPs with high quality genotype data were used in the analysis.

Replication genotyping and quality control

SNPs with p below 10^{-4} in the discovery stage and showing no heterogeneity (I^2 $p > 0.05$) were selected for replication. Genotyping of replication samples from EDOS, FLOS, SALAMANCA, TURIN and SIENA was performed using KASP assays at LGC Ltd (Hoddesdon, Herts, UK). ORCADES and InCHIANTI genotyped their samples locally at Wellcome Trust Clinical Research Facility, Edinburgh, UK, and Laboratory of Neurogenetics, National Institute of Ageing, Bethesda, USA, respectively (using Illumina HumanHap 200v2 array and Illumina Infinium I HumanHap 500 Beadchips, respectively). Samples with call rates below 90% were removed from the study. Quality control check was performed using 45 known genotypes in the replication set, and SNPs with a concordance rate > 90% were selected. Hardy-Weinberg Equilibrium was also tested and samples below the threshold 10^{-7} were removed.

Imputation

Imputation for autosomal SNPs in each case-control cohort was performed using MACH software for pre-phasing and MINIMAC for imputation within the BC|SNPmax software (BC Platforms Ltd). HapMap European (CEU) phased data from phase II, release 22, genome build 36 was used as reference. Association analysis for imputed alleles was performed using MACH2QTL as implemented in GRIMP web interphase [11]. Quality control was performed after imputation excluding markers with minor allele frequency above 99% and below 1%, and SNPs with poor imputation quality ($r^2 < 0.3$).

Association analysis

During the discovery stage each study was independently tested for association using PLINK software [10] (1-degree-of-freedom). Logistic regression models were used to test for association using four principal components as covariates. The 38 SNPs selected for replication were also tested for association using logistic regression. A similar test was also performed using age as covariate.

Meta-analysis

Autosomal SNPs were meta-analysed using fixed-effect, inverse-variance meta-analysis as implemented in METAL software [12]. SNPs detected in fewer than 1,000 individuals were removed from the analysis. We also set a threshold for standard error < 0.01 and minor allele frequency < 0.01. All SNPs below these thresholds were excluded.

Identification of candidate genes

Candidate genes were identified after plotting the GWAS results for regional associations using LocusZoom software [13]. Recombination areas, defined as genomic regions within two recombination peaks, were considered of interest, based on the linkage disequilibrium principle. Genes located within these regions were described as candidate genes.

Expression quantitative trait locus analysis

The expression of candidate genes in bone tissue was investigated in RNA extracted from transiliac bone biopsies derived from 84 Norwegian women as described previously [14]. In summary, bone biopsies were obtained from 27 healthy controls, 18 osteopenic and 39 osteoporotic individuals. Expression profiling was carried out in an Affymetrix HG U133 2.0 plus array and results were imported into Partek Genomic Suite (Partek Inc., St Louis, MO, USA) and normalised using the Robust Multichip Average (RMA) algorithm. Adjustments for batch effects and synthesis times were further performed and probe sets with maximal log2 signal value below 5 were excluded. Participants of this study were genotyped using an Affymetrix 6.0 array and genotypes were imputed using UK10K project and 1000G phase 1 version 3 phased data as reference panel. Genes that were expressed in bone within the recombination area were then tested for association with SNP genotypes by linear regression analysis using PLINK software [10] .

Identification of causal variants

In order to identify potentially functional variants we focused on a linkage disequilibrium block of about 700Kb surrounding the top hit (rs10190845) delimited by a D' threshold of 0.8 or greater as using Haploview software [15] (HapMap V2 release 21, CEU population). We identified additional proxy SNPs for rs10190845 in the region of interest using SNAP proxy ($r^2 \geq 0.7$, 1000 Genomes Pilot 1 reference panel), resulting in a list of 936 variants. Fine mapping was performed using 1000 Genomes phase 3 as reference panel, and logistic association was carried out in PLINK. 878 out of 936 variants were excluded as they were not nominally associated ($p > 0.05$) with the phenotype of vertebral fracture. The remaining 58 candidate SNPs were tested for association with genes expressed in bone using RNA extracted from transiliac biopsies, as described above. SNPs identified as potential eQTLs,

together with the GWAS top hit were evaluated by the R package SuRFR [16]. For the purpose of this analysis, the ALL model of SuRFR was selected on the basis that we wanted to identify all potentially functional variants regardless of the frequency of the susceptibility allele. Exonic variants were analysed *in silico* using a variety of software: PolyPhen-2, SIFT, ESEfinder 3.0, Rescue-ESE 1.0, SNPS3D, EXPASY [17], PANTHER, PSIPRED, and Mutation Taster (reviewed in [18]).

Supplemental Table 1. Characteristics of the study cohorts.

	Vertebral fracture cases				Controls		
Cohort	**Number**	**Age (years)**	**Lumbar Spine T-score**	**Femoral Neck T-score**	**Cohort**	**Number**	**Age**
Discovery							
AROS	299	67.5 ± 7.9	-2.9 ± 1.2	-3.1 ± 1.0	DNBC	1901	29.6 ± 4.2
BARCOS	72	65.6 ± 9.6	N/A	N/A	REGICOR	62	50.8 ± 6.9
CABRIO	256	71.7 ± 8.3	-2.6 ± 1.2	-2.4 ± 1.0	CABRIO-C	263	70.2 ± 8.2
CAIFOS	98	80.5 ± 2.8	-1.6 ± 1.3	-1.9 ± 0.8	WTCCC2	1298	47.0 ± 0.0
AOGC	234	74.5 ± 8.1	-1.8 ± 1.8	-2.0 ± 1.6	WTCCC2		
DOES	33	81.5 ± 7.2	-1.5 ± 1.7	-1.8 ± 1.1	WTCCC2		
EPIC	150	63.5 ± 9.2	N/A	N/A	WTCCC2		
EDOS	371	72.7 ± 8.7	-3.1 ± 1.3	-2.6 ± 1.0	LBC	784	73.2 ± 4.7
SLOVENIA	40	73.6 ± 6.7	N/A	N/A	SLOVENIA-C	32	61.4 ± 5.9
Total	*1553*					*4340*	
Replication							
EDOS	236	74.3 ± 9.3	-2.9 ± 1.4	-2.6 ± 0.9	ORCADES	1101	55.2 ± 13.7
TURIN	86	74.1 ± 8.1	-2.9 ± 1.3	-2.5 ± 1.0	SIENA	271	63.3 ± 6.6
FLOS	282	61.9 ± 9.5	N/A	N/A	InChianti	670	69.1 ± 6.6
SALAMANCA	63	73.8 ± 8.7	-3.1 ± 0.9	-2.6 ± 0.9	SALAMANCA-C	63	69.9 ± 6.9
Total	*667*					*2105*	

Values are mean ± standard deviation and numbers of individuals. N/A: data not available.

Supplemental Table 2. Details of SNPs from discovery sample selected for replication.

Chr	SNP	Position (bp)*	A	AF	OR (95%CI)	p
4	rs7687824	187088056	a	0.30	1.29 (1.18 - 1.42)	$8.53x10^{-8}$
11	rs4937421	111845587	g	0.35	0.80 (0.73 – 0.88)	$3.69x10^{-6}$
2	rs13409720	231255405	a	0.49	0.81 (0.75 - 0.89)	$4.14x10^{-6}$
2	rs778356	233492962	t	0.24	1.26 (1.14 - 1.39)	$5.15x10^{-6}$
11	rs2473904	34172182	c	0.18	1.29 (1.15 – 1.44)	$5.38x10^{-6}$
19	rs2285515	40352290	c	0.38	0.82 (0.75 – 0.89)	$1.09x10^{-5}$
2	rs13424896	233465223	t	0.22	0.78 (0.70 - 0.87)	$1.28x10^{-5}$
6	rs6557164	152044625	t	0.16	1.31 (1.16 - 1.48)	$1.49x10^{-5}$
4	rs6824581	187004404	t	0.09	1.57 (1.28 - 1.92)	$1.57x10^{-5}$
1	rs620336	209778831	t	0.02	2.20 (1.53 - 3.16)	$1.98x10^{-5}$
1	rs12742784	22554953	t	0.20	0.77 (0.69 - 0.87)	$2.09x10^{-5}$
2	rs10190845	112666992	a	0.03	1.70 (1.32 - 2.17)	$2.36x10^{-5}$
15	rs12591031	81854249	g	0.21	1.27 (1.13 – 1.41)	$2.77x10^{-5}$
15	rs12904645	93616356	a	0.21	0.79 (0.71 - 0.88)	$3.01x10^{-5}$
15	rs2290492	90808978	a	0.23	1.24 (1.12 - 1.37)	$3.45x10^{-5}$
1	rs11184882	106715153	c	0.40	0.82 (0.74 – 0.90)	$3.46x10^{-5}$
2	rs10928585	137484482	a	0.08	1.39 (1.19 - 1.63)	$3.71x10^{-5}$
6	rs9359760	88650298	t	0.19	1.25 (1.12 - 1.40)	$3.94x10^{-5}$
1	rs4077413	204845265	c	0.24	0.80 (0.72 – 0.89)	$5.1x10^{-5}$
11	rs7121756	57504473	a	0.29	1.22 (1.11 - 1.35)	$5.2x10^{-5}$
11	rs4430501	4687303	c	0.34	0.82 (0.75 - 0.90)	$5.31x10^{-5}$
11	rs16928809	2893528	a	0.09	0.73 (0.62 - 0.85)	$5.5x10^{-5}$
2	rs777346	166244164	t	0.49	1.20 (1.10 - 1.31)	$5.68x10^{-5}$
13	rs2146819	26895305	t	0.45	1.22 (1.11 - 1.34)	$6.27x10^{-5}$
16	rs12930710	82816767	t	0.08	1.45 (1.21 - 1.74)	$6.45x10^{-5}$
3	rs9839600	70118001	g	0.13	0.75 (0.65 – 0.86)	$6.56x10^{-5}$
3	rs13087574	74928783	a	0.19	0.77 (0.68 - 0.88)	$7.53x10^{-5}$
15	rs12903172	59156502	c	0.36	0.83 (0.75 – 0.91)	$7.73x10^{-5}$
1	rs1360181	68486723	c	0.16	1.26 (1.12 – 1.41)	$8.39x10^{-5}$
6	rs6902885	127422175	t	0.39	1.21 (1.10 - 1.34)	$8.61x10^{-5}$
11	rs527199	134268614	c	0.47	0.84 (0.77 – 0.92)	1.00×10^{-4}

*HapMap phase II, release 22, genome build 36 was used as reference panel

The allele (A) and allele frequency (AF) for each of the variants is shown along with the p value for association, odds ratio (OR) and 95% confidence interval (95% CI).

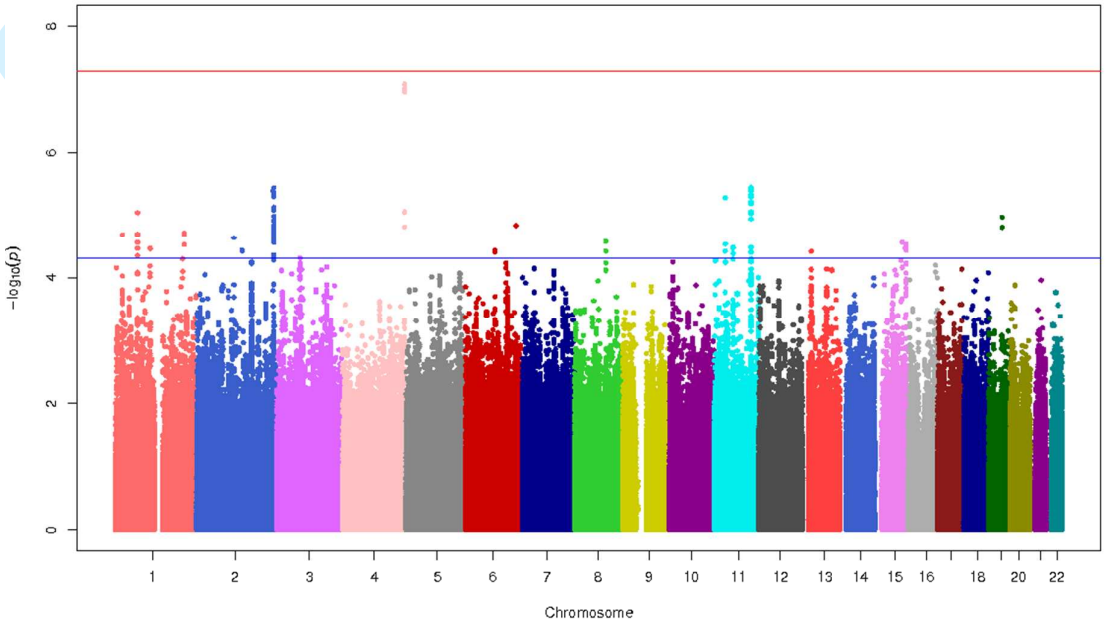
Supplemental Table 3. Details of SNPs found significantly associated with clinical fractures in a previous GWAS [19], also included in the replication stage.

Chr	SNP	Position (bp)	Allele	MAF	OR 95%CI	P
18	rs4796995	13698574	g	0.39	1.08 (1.06 - 1.10)	$8.80x10^{-13}$
7	rs4727338	95958611	g	0.32	1.08 (1.05 - 1.10)	$5.90x10^{-11}$
10	rs1373004	54097831	t	0.13	1.10 (1.06 - 1.13)	$9.00x10^{-9}$
11	rs3736228	67957871	t	0.15	1.09 (1.06 - 1.13)	$1.40x10^{-8}$
4	rs6532023	88992873	t	0.33	1.06 (1.04 - 1.09)	$1.70x10^{-8}$
2	rs4233949	54513211	c	0.37	1.06 (1.04 - 1.08)	$2.60x10^{-8}$
3	rs430727	41103568	t	0.47	1.06 (1.03 - 1.08)	$2.90x10^{-7}$

Supplemental Table 4. Bioinformatic analysis of rs35586251

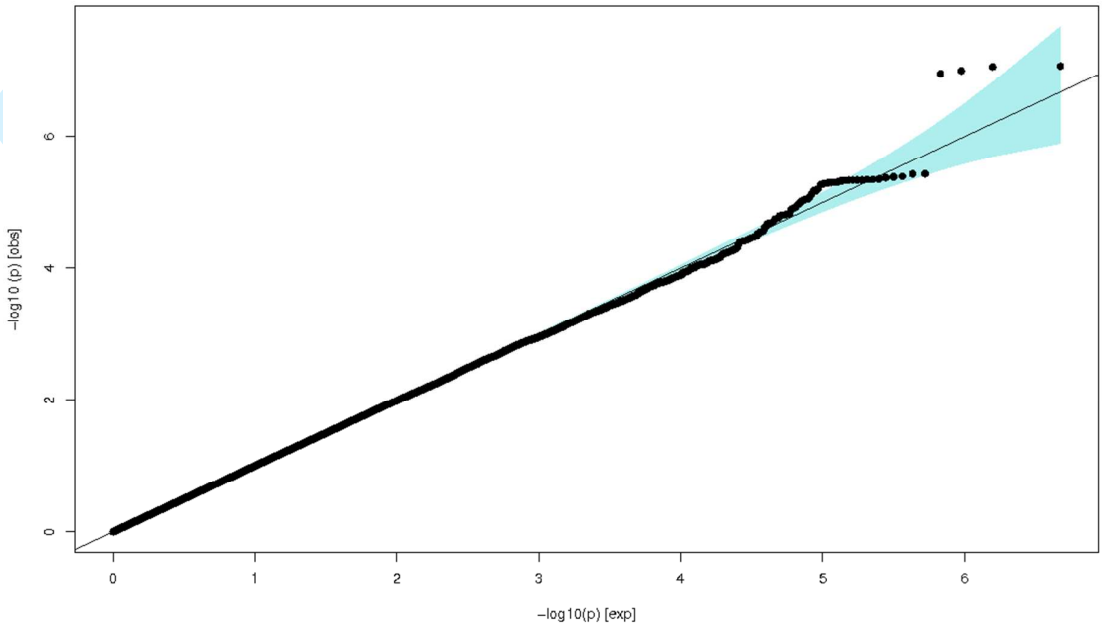
Software	**Score**	**Prediction**
Polyphen	0.854	Possibly damaging
SIFT	0.19	Tolerated
PANTHER	-0.8 (p-value = 0.0998)	Neutral
ESEfinder 3.0		No exonic splicing enhancer binding
Rescue-ESE 1.0		No binding sequence
PSIPRED		SNP located with a predicted b-sheet
SNPS3D	-0.17	Damaging
EXPASY		Hypothetical protein; no domains identified
Mutation Taster	21	Disease causing

Supplemental Figure 1. Loci for susceptibility to clinical vertebral fracture detected by genome wide association.



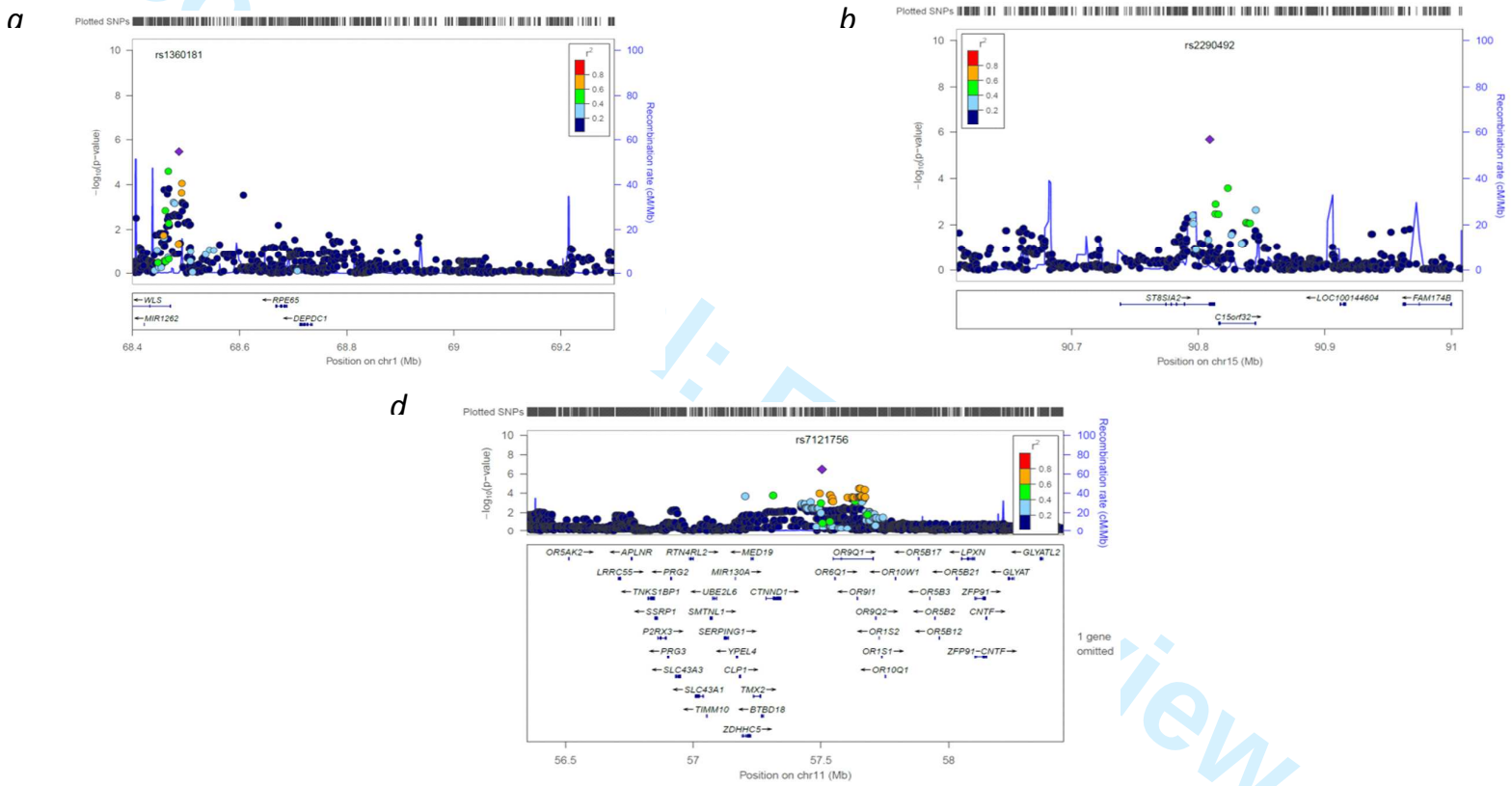
Manhattan plot of association test results from the discovery stage of the GWAS showing the chromosomal position of 2,366,456 genotyped or imputed SNPs plotted against genomic-control–adjusted −log10 *P* values The red horizontal line represents the threshold for genome-wide significance ($p < 5 \times 10^{-8}$) and the blue horizontal line represents the threshold for suggestive association ($p< 5x10^{-6}$).

Supplemental Figure 2. Quantile- quantile plot in the discovery cohort



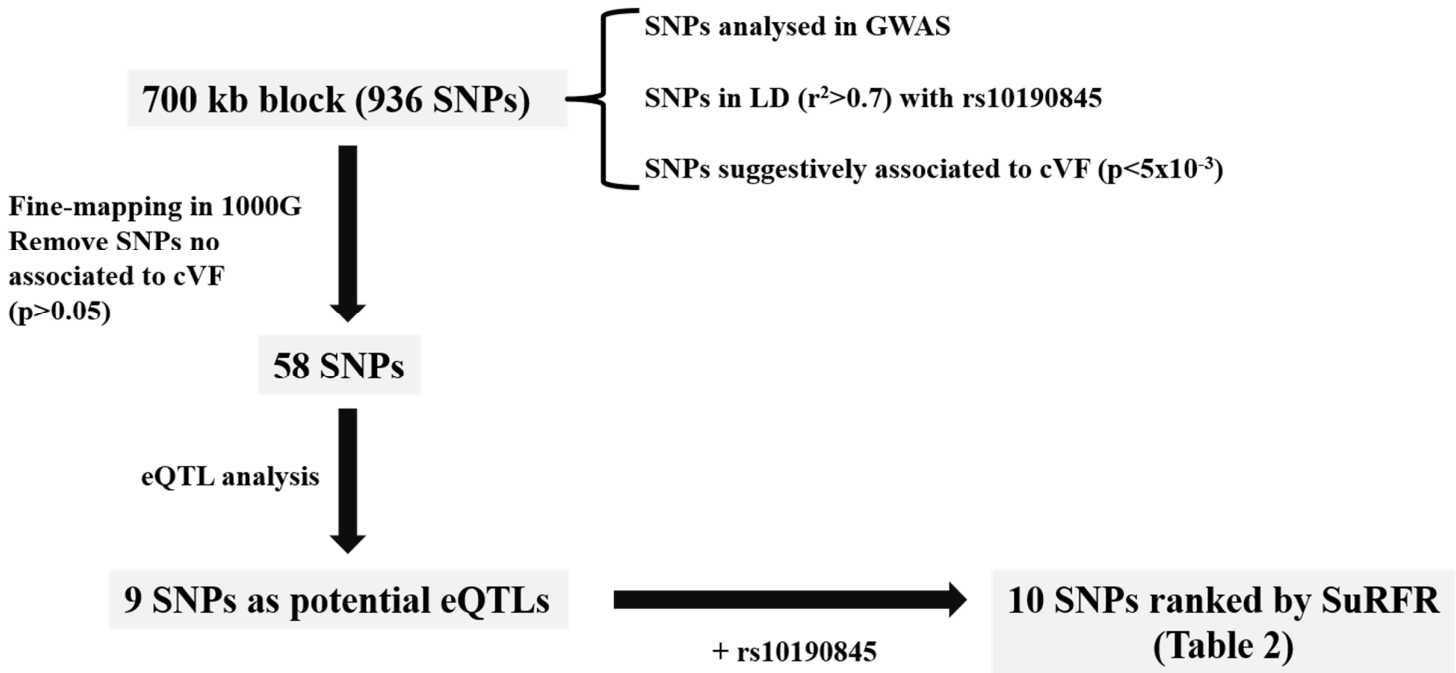
The expected negative logP value is shown on the horizontal axis and the observed negative logP value on the vertical axis

Supplemental Figure 3. Regional association plots for loci suggestively associated with clinical vertebral fractures

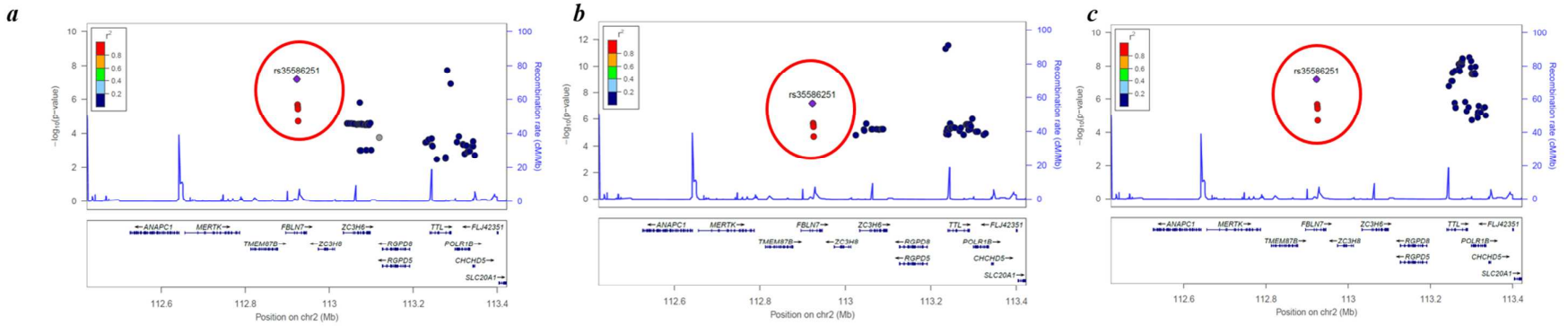


The plots shown are for rs1360181 on chromosome 1 (a); rs2290492 on chromosome 15 (b); and rs7121756 on chromosome 11 (c). In each case the SNPs are colour coded according to the extent of LD with the SNP showing the highest association signal (represented as a purple diamond). The estimated recombination rates (cM/Mb) from HapMap CEU release 22 are shown as light blue lines, and the blue arrows represent known genes in the region.

Supplemental Figure 4. Selection of SNPs within 700 kb around rs10190845 to be ranked by SuRFR software.



Supplemental Figure 5. Regional association plots for SNPs representing an eQTL for *TTL* gene in different tissues.



The plots show eQTLs for *TTL* gene in: a) blood; b) skeletal muscle; c) skin. The SNPs found as eQTLs in the transiliac biopsies were highlighted within the red cicle. Blood eQTL SNPs were extracted from the Blood eQTL browser (http://genenetwork.nl/bloodeqtlbrowser/) [20]; both, skeletal muscle and skin eQTL SNPs were extracted from The Genome-Tissue Expression Project (GTEx) [21,22].

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